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Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies

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This study examined whether antigenic differences among porcine circovirus type 2 (PCV-2) strains could be detected using monoclonal antibodies (mAbs). A subtractive immunization protocol was used for the genotype 2 post-weaning multisystemic wasting syndrome (PMWS)-associated PCV-2 strain Stoon-1010. Sixteen stable hybridomas that produced mAbs with an immunoperoxidase monolayer assay (IPMA) titre of 1000 or more to Stoon-1010 were obtained. Staining of recombinant PCV-2 virus-like particles demonstrated that all mAbs were directed against the PCV-2 capsid protein. Cross-reactivity of mAbs was tested by IPMA and neutralization assay for genotype 1 strains 48285, 1206, VC2002 and 1147, and genotype 2 strains 1121 and 1103. Eleven mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) recognized all strains in the IPMA and demonstrated neutralization of Stoon-1010, 48285, 1206 and 1103, but not VC2002, 1147 and 1121. mAbs 31D5, 48B5, 59C6 and 108E8 did not react with genotype 1 strains or had a reduced affinity compared with genotype 2 strains in the IPMA and neutralization assay. mAb 13H4 reacted in the IPMA with PMWS-associated strains Stoon-1010, 48285, 1206 and VC2002, and the porcine dermatitis and nephropathy syndrome-associated strain 1147, but not with reproductive failure-associated strains 1121 and 1103. mAb 13H4 did not neutralize any of the tested strains. It was concluded that, despite the high amino acid identity of the capsid protein ($\geqslant$91 %), antigenic differences at the capsid protein level are present among PCV-2 strains with a different genetic and clinical background.

INTRODUCTION

Porcine circovirus type 2 (PCV-2) is widespread in domestic and wild pigs. It belongs to the family *Circoviridae*, along with porcine circovirus type 1 (PCV-1), which was discovered and characterized as a non-cytopathic contaminant of the continuous porcine kidney cell line PK-15 (ATCC CCL-33) (Tischer *et al.*, 1974, 1982). PCV-1 is not regarded as a pathogen for pigs (Tischer *et al.*, 1986; Allan *et al.*, 1995), whereas PCV-2 is considered as the crucial pathogen in post-weaning multisystemic wasting syndrome (PMWS), a multifactorial swine disease that causes wasting and death in weaned piglets (Harding, 1996; Nayar *et al.*, 1997; Ellis *et al.*, 1998; Allan & Ellis, 2000). Besides wasting, PCV-2 may also cause reproductive failure (West *et al.*, 1999; Ladekjær-Mikkelsen *et al.*, 2001; Meehan *et al.*, 2001; Sanchez *et al.*, 2001, 2003; Mateusen *et al.*, 2004). PCV-2 has also been isolated from pigs with porcine dermatitis and nephropathy syndrome (PDNS) and a number of other diseases, but neither PDNS nor these other diseases have been reproduced experimentally (Allan *et al.*, 2000; Rosell *et al.*, 2000; Segales *et al.*, 2005, Wellenberg *et al.*, 2004).

The PCV-2 virion measures approximately 17 nm in diameter, is non-enveloped and consists of a circular single-stranded DNA surrounded by an icosahedral capsid (Allan *et al.*, 1998). The ambisense DNA molecule contains about 1.77 kb and 11 putative open reading frames (ORFs) (Hamel *et al.*, 1998). Proteins encoded by three of these ORFs are considered to play a role in the pathogenesis of PCV-2 infections. ORF1 encodes the replication-associated proteins Rep and Rep′ (Mankertz & Hillenbrand, 2001; Cheung, 2003; Mankertz *et al.*, 2003). Rep and Rep′ are 37.5 and 20.2 kDa, respectively. ORF2 encodes the

The GenBank/EMBL/DDBJ accession numbers of the ORF2 sequences from strains 1206, VC2002-k2 and VC2002-k39 determined in this study are EF990644, EF990645 and EF990646, respectively.

27.8 kDa capsid protein (Hamel *et al.*, 1998; Meehan *et al.*, 1998; Morozov *et al.*, 1998; Mankertz *et al.*, 2000; Nawagitgul *et al.*, 2000). The ORF2 protein is the only structural protein. The ORF3 protein has a molecular mass of 11.8 kDa and has recently been associated with apoptosis *in vitro* and with viral pathogenesis in mice (Liu *et al.*, 2005, 2006).

Meerts *et al.* (2005a) demonstrated biological differences among different PCV-2 strains *in vitro*. Replication kinetics of PMWS- and PDNS-associated PCV-2 strains were significantly different from reproductive failure-associated PCV-2 strains. Recently, it was demonstrated that the virulence of a PCV-2 isolate originating from a PMWS-affected animal differed significantly from an isolate recovered from a subclinically infected animal. Important differences in serological profile, virus replication and severity of lesions were shown after experimental inoculation of specific-pathogen-free (SPF) pigs (Opriessnig *et al.*, 2006).

Among various strains of PCV-2, the identity at the nucleotide level of the Rep protein and the capsid protein is 97–100 % and 91–100 %, respectively. At protein level, identity is 97–100 % for Rep and 89–100 % for the capsid protein (Larochelle *et al.*, 2002). Several studies have suggested that genetic differences in PCV-2 are associated with the geographical region from which the isolates originated (Fenaux *et al.*, 2000; Hamel *et al.*, 2000; Mankertz *et al.*, 2000; Kim & Lyoo, 2002) and a recently proposed classification system (Olvera *et al.*, 2007) divides PCV-2 into two genotypes (1 and 2) and eight clusters (1A–1C and 2A–2E). Although several antigenic domains have been discovered on the capsid protein (Mahé *et al.*, 2000; Lekcharoensuk *et al.*, 2004; Olvera *et al.*, 2007), no association has been established so far between the sequence of the capsid and the pathogenicity of a PCV-2 strain (Fenaux *et al.*, 2000; Meehan *et al.*, 2001; Larochelle *et al.*, 2002; Pogranichniy *et al.*, 2002; de Boisséson *et al.*, 2004; Grierson *et al.*, 2004). Until now, mouse monoclonal antibodies (mAbs) directed against PCV-2 have not shown major differences in reactivity to different PCV-2 strains (Allan *et al.*, 1999; McNeilly *et al.*, 2001).

In this study, mAbs to PCV-2 were produced, characterized and used to identify antigenic differences among PCV-2 strains with a different genotype and originating from different clinical presentations.

METHODS

Viruses. Seven different PK-15-adapted PCV-2 strains were used in this study. Their origin and genotype (Cheung *et al.*, 2007; Olvera *et al.*, 2007) are shown in Table 1. The replication kinetics of these strains have been documented previously (Meerts *et al.*, 2005a). PCV-1 originated from the persistently infected PK-15 cell line ATCC CCL-33 (Tischer *et al.*, 1974, 1982). All PCV-2 strains used were passaged 11–17 times, except for strain 1121, which was passaged 30 times.

Recombinant PCV-2 virus-like particles. PCV-2 virus-like particles (VLPs) were obtained by infecting Sf9 insect cells with a baculovirus recombinant P054 expressing the ORF2 of PCV-2 strain Stoon-1010. Purification of VLPs was performed in a caesium chloride gradient as described by Nawagitgul *et al.* (2000).

Cells. PCV-negative PK-15 cells and the persistently PCV-1-infected PK-15 cell line ATCC CCL-33 were grown in minimal essential medium (MEM) containing Earle's salts (Gibco), supplemented with 5 or 10 % fetal bovine serum (FBS), 0.3 mg glutamine ml^{-1}, 100 U penicillin ml^{-1}, 0.1 mg streptomycin ml^{-1} and 0.1 mg kanamycin ml^{-1}. Cell cultures were maintained at 37 °C in the presence of 5 % CO_2.

Mouse immunization. Before immunization, mice were made immunotolerant to PK-15 cells as described by Matthew & Sandrock (1987). Four 6-week-old female BALB/c mice were injected intraperitoneally (i.p.) with 1.5×10^7 PCV-negative PK-15 cells in 300 µl PBS. After 10 min, 24 and 48 h, cyclophosphamide (Sigma) was injected i.p. at a dose of 100 mg $(kg\ body\ weight)^{-1}$ in a total volume of 500 µl PBS. After 3 and 6 weeks, injections with PK-15 cells and cyclophosphamide were repeated. Two weeks after the last treatment, 2.25×10^7 Stoon-1010-inoculated PK-15 cells were injected i.p. in a volume of 300 µl PBS mixed with an equal amount of complete Freund's adjuvant (Sigma). At this time point and 2 weeks later, serum was collected from mice. Three weeks after the inoculation with PCV-2, one mouse received an i.p. injection with 4.5×10^7 Stoon-1010-inoculated PK-15 cells diluted in 600 µl PBS. Euthanasia was performed 4 days later and the spleen was collected.

Production and screening of hybridomas. Hybridoma cells were produced by fusion of spleen cells with SP 2/0 myeloma cells as described by Galfre & Milstein (1981). The resulting hybridoma cells were maintained in RPMI 1640 (Gibco) supplemented with 10 % FBS. PCV-2-specific mAbs in supernatant fluids were demonstrated on

Table 1. Origins of the PCV-2 strains used in this study

Strain	Clinical origin	Genotype	Geographical origin	Reference
Stoon-1010	PMWS-affected piglet	2	Canada	Meehan *et al.* (1998)
48285	PMWS-affected piglet	1	France	Meehan *et al.* (1998)
1206	PMWS-affected piglet	1	Belgium	Meerts *et al.* (2005a)
VC2002	PMWS-affected piglet	1	Belgium	Meerts *et al.* (2004)
1147	PDNS-affected piglet	1	UK	Meehan *et al.* (2001)
1121	Aborted fetuses	2	Canada	Meehan *et al.* (2001)
1103	Aborted fetuses	2	Canada	Meehan *et al.* (2001)

PCV-negative and Stoon-1010-inoculated PK-15 cells by an immunoperoxidase monolayer assay (IPMA) adapted from Labarque *et al.* (2000). After incubation with undiluted supernatant fluids for 1 h at 37 °C, cells were washed twice with PBS. Subsequently, a 1 : 500 dilution of horseradish peroxidase-labelled goat anti-mouse polyclonal antibodies (pAbs) (Dako) in PBS was added for 1 h at 37 °C. After washing twice in PBS, substrate solution was added and cell cultures were analysed by light microscopy (Olympus Optical Co.). Selected hybridoma cultures were cloned by limiting dilution.

Determination of mAb class. The isotype of the produced mAbs was determined using a peroxidase-based commercial mouse mAb identification kit (Zymed). This test identifies the IgG1, IgG2a, IgG2b, IgG3, IgA and IgM isotype classes and the κ and λ type of the light chain using monospecific rabbit pAbs. Supernatant fluids of anti-PRV mAbs 13D12 (IgG1) and 1C11 (IgG2a) (Nauwynck & Pensaert, 1995) and anti-*Escherichia coli* mAb E7G3 (IgG3) (Tiels *et al.*, 2007) were used as positive controls.

Indirect immunofluorescence staining of recombinant PCV-2 VLPs. The VLP staining technique was adapted from Misinzo *et al.* (2005). Briefly, purified VLPs were diluted 1 : 100 in PBS, smeared onto microscope slides, air dried and fixed with 3 % (w/v) paraformaldehyde in PBS for 10 min at room temperature. Fixed VLPs were incubated with undiluted hybridoma supernatants for 1 h at 37 °C, followed by incubation with a 1 : 500 dilution of fluorescein isothiocyanate-labelled goat anti-mouse pAbs (Molecular Probes) containing 10 % PCV-2-negative goat serum for 1 h at 37 °C. mAb F217 (McNeilly *et al.*, 2001) diluted 1 : 50 in PBS was used as a positive control. mAbs 13D12 and 1C11 were included as negative controls. A Leica DM/RBE fluorescence microscope (Leica Microsystems) was used for visualization.

Western blot analysis. Stoon-1010-inoculated and mock-inoculated PCV-negative PK-15 cells were harvested by scraping. Cells were pelleted by centrifugation at 15 700 ***g*** for 20 min at 4 °C and subsequently lysed for 1 h at 37 °C in TNE [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA] containing 1 % NP-40 (Roche), protease inhibitors (Complete; Roche) and 0.5 % SDS. Cells were centrifuged at 15 700 ***g*** for 10 min at 4 °C and resuspended in a non-reducing Laemmli buffer. This mixture was boiled for 5 min and stored at −20 °C until use. Proteins were separated by standard SDS-PAGE (Laemmli, 1970) and transferred to a PVDF membrane (Amersham Biosciences). This membrane was then incubated for 1 h at room temperature in PBS containing 0.1 % Tween 20 (PBS-Tween), supplemented with 5 % BSA (Sigma). After washing in PBS-Tween, membranes were incubated overnight at 4 °C with a 1 : 5 dilution of the mAbs in PBS-Tween. mAb F190 (McNeilly *et al.*, 2001) and biotinylated purified porcine pAbs, originating from a PCV-2-negative SPF pig inoculated with strain 1121 (Pensaert *et al.*, 2004; Meerts *et al.*, 2005a), were used as positive controls. mAbs 13D12 and 1C11 were included as negative controls. Next, a 1 : 300 dilution of biotinylated sheep anti-mouse pAbs and a 1 : 300 solution of a streptavidin–biotinylated horseradish peroxidase complex (Amersham Biosciences) was applied. Membranes were washed twice with PBS-Tween between incubations. Antigen–antibody complexes were visualized using an enhanced chemiluminescence assay (Amersham Biosciences).

Reactivity of mAbs to different PCV-2 strains. PCV-2 strains Stoon-1010, 48285, 1206, VC2002, 1147, 1121 and 1103 were used to prepare 96-well IPMA plates as described by Labarque *et al.* (2000). PCV-negative PK-15 cells and the persistently PCV-1-infected PK-15 cell line were used for control IPMA plates. The staining procedure was similar to the IPMA technique described above. Tenfold dilutions of hybridoma supernatants were prepared in PBS and used as primary antibodies. IPMA antibody titres of a hybridoma supernatant were expressed as the reciprocal of the last dilution that resulted in a positive reaction. These assays were performed three times for each strain.

Sensitive neutralization assays. In order to detect the neutralizing activity of the mAbs, a sensitive neutralization assay was adapted from the method of Meerts *et al.* (2005b). Briefly, $10^{4.3}$ $TCID_{50}$ PCV-2 in a volume of 200 µl was incubated for 1 h at 37 °C with 200 µl undiluted hybridoma supernatant. After incubation, this mixture was added to semi-confluent monolayers of PCV-negative PK-15 cells in four wells of a 96-well plate. After 1 h at 37 °C, cell cultures were washed twice in MEM and fresh medium was added. Cell cultures were fixed 36 h later. At this time point, the first replication cycle of PCV-2 was completed (Meerts *et al.*, 2005a). PCV-2-infected PK-15 cells were stained by an IPMA using porcine PCV-2-specific pAbs, originating from a Stoon-1010-inoculated gnotobiotic pig. The number of infected cells per well was determined by light microscopy. The neutralizing activity of a hybridoma supernatant was expressed as the percentage reduction in the number of infected cells in comparison with medium. Assays were performed with all seven strains. Anti-PCV-2 mAb F190 was used as a positive control. mAbs 13D12 and 1C11 were used as negative controls. A mAb was considered as neutralizing when its mean neutralizing activity was higher than the mean neutralizing activity + SD of the negative controls. Sensitive neutralization experiments were performed three times for each strain.

Sequencing of ORF2 from strains 1206 and VC2002. The Belgian PCV-2 strains 1206 and VC2002 were purified by ultracentrifugation at 180 000 ***g*** for 3 h through a 30 % sucrose gradient as described by Delputte *et al.* (2002). A set of PCR primers was designed based on alignment of the genome sequences of strains Stoon-1010, 48285, 1147, 1121 and 1103. The primer set PCV-2-FW (5′-AGCGCAC-TTCTTTCGTTTTCAG-3′) and PCV-2-REV (5′-GAATGCGGCCG-CTTATCACTTCGTAATGGTTTTTATTATTCA-3′) amplified the complete ORF2. Two internal oligonucleotides were synthesized: CV1 (5′-GGGCTGTGGCCTTTGKTAC-3′) and CV2 (5′-TGTR-GACCACGTAGGCCTCG-3′). These internal oligonucleotides were as described by Fenaux *et al.* (2000) with minor modifications, and were used for sequencing. A 1 : 200 fraction of proteinase K-treated ultrapurified PCV-2 was used as template in PCRs using Platinum *Pfx* DNA polymerase (Invitrogen) at an annealing temperature of 60 °C and using the cycling conditions described by the manufacturer. PCR products (~800 bp) were treated with exonuclease I and antarctic phosphatase (New England Biolabs) and used directly for cycle sequencing with a Big Dye Terminator Cycle Sequencing kit v1.1 (Applied Biosystems) and PCV-2 primers. Cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems). Additionally, PCR products (~800 bp) were gel purified using a QIAquick gel extraction kit (Qiagen) and cloned in pBluescript II SK(+) cut with *Eco*RV and treated with antarctic phosphatase. Clones containing the PCV-2 ORF2 were sequenced using T7 and T3 primers as described above. The sequences were analysed and compiled using Align, LAlign, CLUSTAL W and Sixframe in the Biology Workbench (http://workbench.sdsc.edu) and Align2sequences, BLASTN and BLASTP at www.ncbi.nlm.nih.gov. Phylogenetic relationships among sequences were analysed as described by Tripathi & Sowdhamini (2006). Briefly, phylogenetic trees were derived from multiple sequence alignments with PHYLIP v3.67. Bootstrapping was performed 100 times using SEQBOOT. Pairwise distances between genomic sequences and protein sequences were determined with DNADIST and PROTDIST, respectively. Neighbour-joining (NJ) trees were calculated with neighbour and maximum-likelihood (ML) trees with DNAML and ProML. Majority rule consensus trees were obtained with CONSENSE and visualized with DRAWGRAM.

The ORF2 sequences (702 nt, from ATG to the stop codon) from strains 1206, VC2002-k2 and VC2002-k39 have been deposited in

GenBank under accession numbers EF990644, EF990645 and EF990646, respectively.

RESULTS

Mouse immunization

Prior to immunization, four BALB/c mice were made immunotolerant to PK-15 cells by repeated injection of PCV-negative PK-15 cells and cyclophosphamide. After this treatment, no or little reaction to PK-15 cells was observed in IPMAs. All serum samples taken before immunization were negative for anti-PCV-2 antibodies as determined by IPMA. Two weeks after the first immunization, all mice had anti-Stoon-1010 antibody titres of between 2560 and 40 960. One mouse with an IPMA titre of 10 240 and without reaction to PK-15 cells was selected. It received a boost injection 1 week later and its spleen was used for the production of hybridomas.

Production and screening of hybridomas

Forty-four hybridomas that produced mAbs against PCV-2-infected PK-15 cells were frozen. Cloning by limiting dilution resulted in 16 stable hybridomas that produced mAbs with an IPMA titre of 1000 or more to Stoon-1010.

Determination of mAb class

A commercial identification kit was used to determine the isotypes of the mAbs. The results are presented in Table 2. Six hybridomas produced IgG1 mAbs and eight hybridomas produced IgG2a mAbs. mAb 21C12 had an IgG3 isotype. The isotype of mAb 48B5 could not be determined. All mAbs, including mAb 48B5, had a light chain of the κ type.

Indirect immunofluorescence staining of recombinant PCV-2 VLPs

The reactivity of the mAbs to VLPs was tested by performing indirect immunofluorescence staining of VLPs smeared onto glass slides. All 16 mAbs reacted with the VLPs, indicating that the mAbs were directed against the PCV-2 capsid protein. No staining was observed with irrelevant mAbs.

Western blot analysis

The reactivity of the mAbs to Stoon-1010-inoculated PK-15 cells was determined in a Western blot assay. mAbs 31D5, 38C1 and 108E8 gave a strong and specific reaction with a protein of approximately 28 kDa (Fig. 1). For mAb 21C12, a faint but specific band was observed at 28 kDa

Table 2. Isotype and IPMA antibody titres of hybridoma supernatants

IPMA antibody titres of a hybridoma supernatant were expressed as the reciprocal of the last dilution that resulted in a positive reaction.

mAb	Isotype	IPMA antibody titres							
		1010 PMWS Genotype 2	48285 PMWS Genotype 1	1206 PMWS Genotype 1	VC2002 PMWS Genotype 1	1147 PDNS Genotype 1	1121 Abortion Genotype 2	1103 Abortion Genotype 2	PCV-1
9C3	IgG1	10 000	10 000	10 000	10 000	10 000	10 000	10 000	Negative
13H4	IgG2a	1 000	1 000	1 000	100	100	Negative	Negative	Negative
16G12	IgG2a	10 000	10 000	10 000	10 000	10 000	10 000	10 000	Negative
21C12	IgG3	1 000	1 000	1 000	1 000	1 000	1 000	1 000	Negative
31D5	IgG1	10 000	Negative	Negative/1 000*	Negative	Negative	10 000	10 000	Negative
38C1	IgG1	100 000	10 000	100 000	100 000	100 000	100 000	100 000	Negative
43E10	IgG1	1 000	1 000	1 000	1 000	1 000	1 000	1 000	Negative
48B5	No reaction	1 000	Negative	Negative/100*	Negative	Negative	1 000	100	Negative
55B1	IgG2a	10 000	1 000	10 000	10 000	10 000	10 000	10 000	Negative
59C6	IgG2a	10 000	10	100/10 000*	100	100	10 000	10 000	Negative
63H3	IgG2a	10 000	1 000	1 000	1 000	10 000	10 000	10 000	Negative
70A7	IgG2a	10 000	1 000	1 000	10 000	10 000	10 000	1 000	Negative
94H8	IgG2a	10 000	10 000	10 000	10 000	10 000	10 000	10 000	Negative
103H7	IgG2a	10 000	1 000	10 000	10 000	10 000	10 000	10 000	Negative
108E8	IgG1	1 000	10	Negative/1 000*	1	1	1 000	1 000	Negative
114C8	IgG1	1 000	1 000	1 000	1 000	1 000	1 000	1 000	Negative

*mAbs 31D5, 48B5, 59C6 and 108E8 stained two different populations of infected cells in strain 1206. IPMA titres for the first population (~99 % of the infected cells, on the left of the slash) were comparable to those of the genotype 1 strains 48285, VC2002 and 1147. IPMA titres for the second population (~1 % of the infected cells, on the right of the slash) were comparable to those of the genotype 2 strains 1010, 1121 and 1103. These populations were determined by counting the number of infected cells per well after staining with different dilutions of the mAbs.

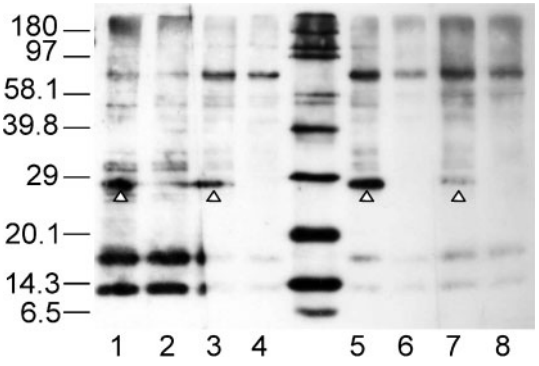

Fig. 1. Western blot analysis of PCV-2 Stoon-1010-inoculated and mock-inoculated PK-15 cells. Odd numbers represent Stoon-1010-inoculated cell lysates, whilst even numbers show mock-inoculated cell lysates. Lanes: 1 and 2, mAb F190 (positive control); 3 and 4, mAb 31D5; 5 and 6, mAb 38C1; 7 and 8, mAb 108E8. All four mAbs reacted specifically with a 28 kDa protein (arrowheads).

(not shown). None of the other mAbs showed reactivity in the Western blot assay.

Reactivity of mAbs to different PCV-2 strains

An IPMA was used to examine the reactivity of hybridoma supernatants to seven different PCV-2 strains (Table 2). Eleven out of 16 hybridomas stained all seven strains with a maximum tenfold variation in titres among the strains (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8). mAbs 31D5, 48B5, 59C6 and 108E8 did not react with the genotype 1 strains 48285, VC2002 and 1147, or they had IPMA antibody titres to these strains that were at least 100 times lower than for the genotype 2 strains Stoon-1010, 1121 and 1103. These four mAbs stained two different populations of infected cells in strain 1206. IPMA antibody titres for the first population (~99 % of the infected cells) were comparable to those of the other genotype 1 strains. IPMA antibody titres for the second population (~1 % of the infected cells) were comparable to those of the genotype 2 strains. These populations were determined by counting the number of infected cells per well after staining with different dilutions of the mAbs. mAb 13H4 stained all four PMWS-associated strains (Stoon-1010, 48285, 1206 and VC2002) and the single PDNS-associated strain (1147), but did not react with the two reproductive failure-associated strains (1121 and 1103). None of the 16 mAbs reacted with PCV-1 or with PK-15 cells.

Sensitive neutralization assays

A sensitive neutralization assay was used to determine the neutralizing activity of hybridoma supernatants. Table 3 shows the percentage neutralization ± SD of the different mAbs. The neutralizing activities of mAbs 13D12 and 1C11 were 7 ± 19 and −1 ± 14 %, respectively. As the mean neutralizing activity of mAb 13D12 + SD was 7 + 19 = 26 %,

Table 3. Neutralizing activity of hybridoma supernatants

The neutralizing activity of a hybridoma supernatant was expressed as the percentage reduction in the number of infected cells in comparison with medium. A mean neutralizing activity of 30 % or more was considered to be neutralization (indicated in bold).

mAb	Neutralization (± SD) (%)						
	1010 PMWS Genotype 2	48285 PMWS Genotype 1	1206 PMWS Genotype 1	VC2002 PMWS Genotype 1	1147 PDNS Genotype 1	1121 Abortion Genotype 2	1103 Abortion Genotype 2
9C3	**94** ± 3	**92** ± 5	**54** ± 10	1 ± 13	**34** ± 20	11 ± 12	**54** ± 16
13H4	−3 ± 14	8 ± 15	10 ± 14	12 ± 12	−4 ± 15	7 ± 11	6 ± 16
16G12	**91** ± 3	**90** ± 6	**41** ± 14	6 ± 18	19 ± 15	−4 ± 9	26 ± 11
21C12	**94** ± 1	**82** ± 8	**44** ± 14	**32** ± 16	0 ± 19	−12 ± 18	**56** ± 21
31D5	**92** ± 2	11 ± 13	6 ± 9	−4 ± 8	6 ± 20	3 ± 12	**54** ± 20
38C1	**95** ± 1	**94** ± 2	**42** ± 17	20 ± 10	**30** ± 20	−13 ± 13	**61** ± 16
43E10	**79** ± 2	**89** ± 2	**38** ± 18	2 ± 4	22 ± 20	6 ± 2	**56** ± 12
48B5	**98** ± 0	19 ± 6	16 ± 5	−5 ± 3	−7 ± 7	23 ± 22	**49** ± 13
55B1	**84** ± 2	**94** ± 2	**55** ± 16	−2 ± 3	25 ± 12	6 ± 4	**32** ± 15
59C6	**72** ± 3	**30** ± 9	17 ± 13	4 ± 9	4 ± 10	6 ± 16	28 ± 20
63H3	**72** ± 4	**77** ± 3	**30** ± 16	0 ± 5	10 ± 8	−14 ± 18	**50** ± 17
70A7	**88** ± 2	**89** ± 2	**57** ± 17	10 ± 1	21 ± 12	−9 ± 10	**39** ± 16
94H8	**62** ± 14	**82** ± 4	**49** ± 14	6 ± 5	16 ± 12	2 ± 2	**56** ± 8
103H7	**51** ± 6	**62** ± 9	**32** ± 12	5 ± 12	8 ± 22	3 ± 14	**55** ± 19
108E8	**88** ± 2	**35** ± 11	9 ± 9	8 ± 3	2 ± 9	−6 ± 14	**67** ± 5
114C8	**71** ± 4	**74** ± 7	**39** ± 23	14 ± 4	20 ± 9	−10 ± 17	**51** ± 5

a mAb was arbitrarily considered as neutralizing when its mean neutralizing activity was higher than 30 %. The 11 mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) that reacted equally with all seven PCV-2 strains in the IPMA demonstrated neutralization of Stoon-1010 (up to 95 %), 48285 (up to 94 %), 1206 (up to 57 %) and 1103 (up to 61 %). The four mAbs (31D5, 48B5, 59C6 and 108E8) that had a higher affinity for genotype 2 strains than for genotype 1 strains in the IPMA demonstrated neutralization of the genotype 2 strains Stoon-1010 (up to 98 %) and 1103 (up to 67 %). For these four mAbs, neutralization of genotype 1 strains 48285 and 1206 was absent or very low (up to 35 %). mAb 13H4 did not neutralize any of the seven tested strains. Only one mAb (21C12) demonstrated some neutralization (32 %) of strain VC2002 and only two mAbs (9C3 and 38C1) demonstrated some neutralization (34 and 30 %, respectively) of strain 1147. None of the 16 mAbs neutralized strain 1121.

Sequencing of ORF2 from strains 1206 and VC2002

The ORF2 of the Belgian PMWS-associated PCV-2 strains 1206 and VC2002 was amplified by PCR and sequenced. Strain 1206 contained an ORF2 of 702 bp (starting from ATG and including the stop codon) encoding a 233 aa protein. Sequencing of the VC2002 ORF2 PCR product resulted in a sequence containing ambiguities at different positions. Therefore, the VC2002 PCR fragment was cloned in pBluescript II SK(+) and 12 clones were sequenced. Clone VC2002-k39 contained an ORF of 702 bp (starting from ATG and including the stop codon) encoding a protein of 233 aa. Ten other VC2002 clones were almost 100 % identical at the nucleotide level with clone k39 with between 1 and 3 nt differences. Clone VC2002-k2 showed 94 % identity with k39 at the nucleotide and amino acid levels and 96–99 % amino acid identity with strains from China (e.g. NCBI protein database accession nos AAP44186, AAU87508 and AAT97651), The Netherlands (accession no. AAS65982; Grierson *et al.*, 2004) and a strain isolated from wild boars in Germany (accession no. AAU13781; Knell *et al.*, 2005). Capsid protein similarity among the seven different strains used in this study was determined using pairwise alignments and CLUSTAL W (Fig. 2). The ORF2 amino acid identities of the strains used in this study is shown in Table 4. Fig. 3 shows a phylogenetic tree of the ORF2 protein based on the NJ method with the percentage confidence shown on each branch. This tree was constructed with ORF2 protein sequences from this study and sequences chosen from the different clusters from Olvera *et al.* (2007). The latter sequences are shown in Table 5. Genotype 1 strains 48285, 1206, VC2002-k39 and 1147 were assigned to cluster 1A/1B, VC2002-k2 to cluster 1C and genotype 2 strains Stoon-1010, 1121 and 1103 to cluster 2E. The same strain classification was obtained by using the ML method and with ORF2 DNA sequences.

```
1206         MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTIKRTTVRTPSWAVDMMRFNINDFL 80
1147         MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTIKRTTVKTPSWAVDMMRFNINDFL 80
48285        MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRTTVKTPSWAVDMMRFNINDFL 80
VC2002-k39   MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRTTVRTPSWAVDMMRFNINDFL 80
VC2002-k2    MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTIGYTVKATTVRTPSWNVDMMRFNINDFL 80
1121         MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDDFV 80
1103         MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDDFV 80
Stoon-1010   MTYPRRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRKNGIFNTRLSRTFGYTVKRTTVTTPSWAVDMMRFKIDDFV 80
             ************************************************:***:* *** **** ******:*:**:

1206        PPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHSRY 160
1147        PPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHSRY 160
48285       PPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTITQPFSYHSRY 160
VC2002-k39  PPGGGSNPRSVPFEYYRIRKVKVEFWPCSPITQGDRGVGSSAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHSRY 160
VC2002-k2   PPGGGSNPLTVPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKANALTYDPYVNYSSRHTIPQPFSYHSRY 160
1121        PPGGGTNKISIPFEYYRIKKVKVEFWPCSPITQGDRGVGSTAVILDDNFVPKATALTYDPYVNYSSRHTIPQPFSYHSRY 160
1103        PPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVPKATAQTYDPYVNYSSRHTIPQPFSYHSRY 160
Stoon-1010  PPGGGTNKISIPFEYYRIRKVKVEFWPCSPITQGDRGVGSTAVILDDNFVTKATALTYDPYVNYSSRHTIPQPFSYHSRY 160
            *****:*  ::*******:*********************:*********.**.* **************.*********

1206        FTPKPVLDSTIDYFQPNNKRNQLWLRLQTAGNVDHVGLGIAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP 233
1147        FTPKPVLDSTIDYFQPNNKRNQLWLRLQAAGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP 233
48285       FTPKPVLDSTIDYFQPNNKRNQLWLRLQTTGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP 233
VC2002-k39  FTPKPVLDSTIDYFQPNNKRNQLWLRLQTAGNVDHVGLGTAFENSIYDQEYNIRVTMYVQFREFNLKDPPLNP 233
VC2002-k2   FTPKPVLDRTIDYFQPNNKRNQLWLRLQTTGNVDHVGLGTAFENSKYDQDYNIRITMYVQFREFNLKDPPLNP 233
1121        FTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTMYVQFREFNLKDPPLNP 233
1103        FTPKPVLDSTIDYFQPNNKRNQLWLRLQTSRNVDHVGLGTAFENSKYDQDYNIRVTLYVQFREFNLKDPPLNP 233
Stoon-1010  FTPKPVLDSTIDYFQPNNKRNQLWLRLQTSGNVDHVGLGAAFENSKYDQDYNIRVTMYVQFREFNLKDPPLKP 233
            ******** ********************:: ******** ***** ***:****:*:***************:*
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Fig. 2. ORF2 amino acid alignment of the PCV-2 strains used in this study. GenBank accession numbers are: EF990644 (1206), AJ293869 (1147), AF055394 (48285), EF990646 (VC2002-k39), EF990645 (VC2002-k2), AJ293868 (1121), AJ293867 (1103) and AF055392 (Stoon-1010). An asterisk indicates a single, fully conserved residue, a colon indicates conservation of strong groups, and a dot represents conservation of weak groups; spaces indicate no consensus (amino acids shown in bold).

Table 4. ORF2 amino acid identity within PCV-2 strains used in this study

The percentage amino acid identities given are the result of pairwise alignments of the ORF2 proteins. Percentage identities between the genotype 1 strains (except VC2002-k2) are shown in bold; percentage identities between the genotype 2 strains are shown in bold and italics; percentage identities between the VC2002-k2 strain and other strains are underlined.

Strain	1206	1147	48285	VC2002-k39	1121	1103	1010	VC2002-k2
1206	100	**98**	**98**	**98**	92	91	93	93
1147		100	**98**	**98**	92	91	92	93
48285			100	**98**	93	92	93	94
VC2002-k39				100	93	93	93	94
1121					100	***98***	***97***	91
1103						100	***97***	91
1010							100	92
VC2002-k2								100

DISCUSSION

This is the first study to demonstrate antigenic diversity among PCV-2 strains. This was established by the production and characterization of mAbs directed to the PCV-2 capsid protein. The cross-reactivity of the mAbs to seven different PCV-2 strains with a different genotype and originating from various clinical conditions was determined.

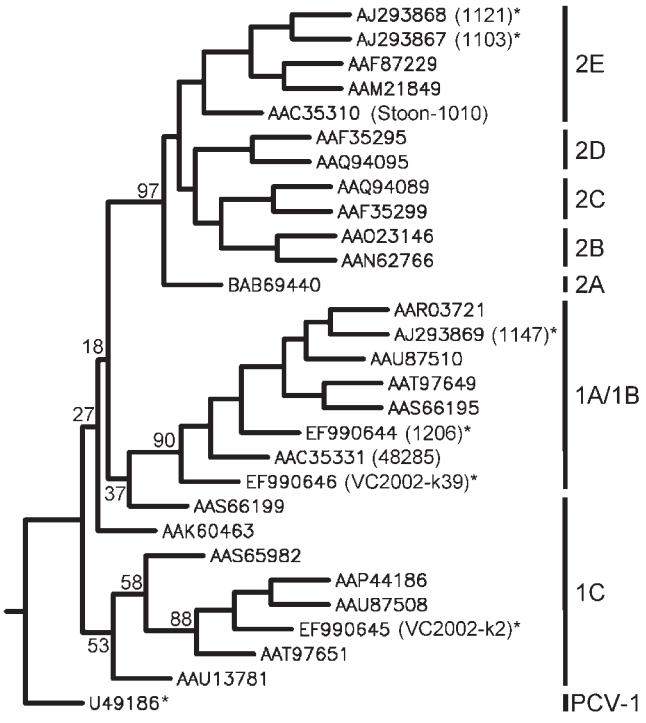

Fig. 3. Unrooted phylogenetic tree constructed using the NJ method. The percentage confidence is indicated on the branches. This tree was based on the ORF2 protein sequences of the PCV-2 strains that were used in the present study (strain names in parentheses), one PCV-1 sequence (outgroup) and 20 PCV-2 sequences that were obtained from Olvera *et al.* (2007). These sequences are listed in Table 5. *, No NCBI protein accession number was available for the ORF2 protein, so the GenBank nucleotide sequence was used.

Eleven mAbs (9C3, 16G12, 21C12, 38C1, 43E10, 55B1, 63H3, 70A7, 94H8, 103H7 and 114C8) reacted equally with the seven PCV-2 strains used in the IPMA. Four other mAbs (31D5, 48B5, 59C6 and 108E8) were able to differentiate the genotype 1 strains 48285, 1206, VC2002 and 1147 from the genotype 2 strains Stoon-1010, 1121 and 1103 by IPMA, as they did not react with genotype 1 strains or had a reduced affinity compared with genotype 2 strains. The IPMA results of the latter four mAbs were also reflected in the neutralization assays. Until now, mAbs have not allowed differentiation of PCV-2 strains (Allan *et al.*, 1999; McNeilly *et al.*, 2001). mAbs 31D5, 48B5, 59C6 and 108E8 also did not react with or had a reduced affinity for tissue sections originating from the Belgian PMWS-affected pig from which the VC2002 strain was isolated. This was demonstrated by immunofluorescence staining and suggests that the results obtained by IPMA for mAbs 31D5, 48B5, 59C6 and 108E8 were not a consequence of PCV-2 cell culture adaptation (data not shown).

Using the IPMA, mAbs 31D5, 48B5, 59C6 and 108E8 stained two different populations of infected cells in strain 1206. This suggests that strain 1206 consists of two viral subpopulations, where 99 % of the virus behaves as a genotype 1 strain and 1 % of the virus behaves as a genotype 2 strain. No signs of the existence of subpopulations were detected by sequencing of strain 1206. This may be explained by the fact that the putative genotype 2 subpopulation was present at a very low level (1 %). Sequencing of the VC2002 strain did reveal the existence of two PCV-2 subpopulations in the virus stock. After cloning, two distinct sequences were derived from strain VC2002. Phylogenetic analysis assigned clone VC2002-k39 to cluster 1A/1B and demonstrated clustering of clone VC2002-k2 with strains from China, The Netherlands (Grierson *et al.*, 2004) and a strain isolated from German wild boars (Knell *et al.*, 2005), which documents the putative epidemiological link between PCV-2 infections in

Table 5. Name, phylogenetic cluster (according to Olvera *et al.*, 2007) and origin of the ORF2 sequences used in Fig. 3

NCBI protein accession no.	GenBank accession no.	Strain	Cluster	Clinical origin	Geographical origin	Reference
–	U49186	pPCV-PSTI	PCV-1	PK-15 cell line	UK	Meehan *et al.* (1997)
AAT97649	AY686764	ZJ	1A	Unknown	China	
AAR03721	AY424404	AUT4	1A	Unknown	Austria	
AAU87510	AY682992	CHST	1B	Unknown	China	
AAS66195	AY556475	GX	1B	PMWS	China	
AAK60463	AY035820	–	1C	PMWS	China	Song *et al.* (2007)
AAS66199	AY556477	HuNan	1C	PMWS	China	
AAP44186	AY291317	HB	1C	PMWS	China	
AAU87508	AY682991	CHL	1C	Unknown	China	
AAT97651	AY686765	JXIII	1C	Unknown	China	
AAS65982	AY484410	Nl-Control-4	1C	Subclinical	Netherlands	Grierson *et al.* (2004)
AAU13781	AY713470	Wild boar	1C	Subclinical	Germany	Knell *et al.* (2005)
BAB69440	AB072302	No. 26	2A	PMWS	Japan	
AAO23146	AY180396	Pingtung-4	2B	Unknown	Taiwan	
AAN62766	AY146991	Pingtung-1	2B	Unknown	Taiwan	
AAQ94089	AY256455	212	2C	PDNS	Hungary	Dán *et al.* (2003)
AAF35299	AF201308	SPA1	2C	PMWS	Spain	Mankertz *et al.* (2000)
AAQ94095	AY256458	326	2D	PMWS	Hungary	Dán *et al.* (2003)
AAF35295	AF201306	GER2	2D	PMWS	Germany	Mankertz *et al.* (2000)
AAM21849	AY094619	688	2E	PMWS	Canada	Cheung (2003)
AAF87229	AF264039	26607	2E	PMWS	USA	Fenaux *et al.* (2000)

domestic and wild pigs (Cságola *et al.*, 2006). The identification of two different PCV-2 sequences in one animal has been reported previously (de Boisséson *et al.*, 2004; Opriessnig *et al.*, 2006; Cheung *et al.*, 2007), but the role of multiple PCV-2 infections in the pathogenesis of PCV-2-associated diseases is not clear.

Using protein sequences (NJ and ML methods), we were not able to differentiate between clusters 1A and 1B, and not all sequences that were previously classified as 1C (Olvera *et al.*, 2007) were found in the 1C cluster. Using the corresponding DNA sequences (NJ and ML methods), the same topology was obtained as by Olvera *et al.* (2007), with the only difference being that clusters 1A and 1B could not be differentiated in the present study (data not shown). We assume that these differences are a consequence of the reduced number of sequences used.

Putative amino acid substitutions that discriminate the genotype 1 strains 48285, 1206, VC2002-k39 and 1147 from the genotype 2 strains Stoon-1010, 1121 and 1103 are located at positions 63, 88, 89 and 206. At position 63, a threonine was substituted for a lysine or an arginine. At position 88, a lysine was replaced by a proline, and at position 89, an isoleucine was replaced by an arginine. These three substitutions all involve the basic amino acids lysine and arginine. Due to the differences in size, charge and hydrophobicity between lysine/arginine and threonine, proline and isoleucine, this may have major consequences on the secondary and tertiary structure of the PCV-2 capsid protein. The same applies to position 206, where a lysine was replaced by an isoleucine. Linear antigenic determinants of the PCV-2 ORF2 protein, as determined by PEPSCAN, are located at positions 65–87, 113–139, 169–183 and 193–207 (Mahé *et al.*, 2000). Positions 63, 88 and 89, where non-conserved mutations were found in the present study, are located at the outer borders of linear epitope 65–87, whereas position 206, where another non-conserved mutation was found, is located at the inner border of linear epitope 193–207. Therefore, we speculate that the amino acid substitutions that involve basic amino acids at positions 63, 88, 89 and 206 might be responsible for the fact that mAbs 31D5, 48B5, 59C6 and 108E8 did not react with the genotype 1 strains or that they had a reduced affinity for these strains in the IPMA and neutralization assay.

This study also demonstrated that mAb 13H4 did not react specifically with the reproductive failure-associated strains 1121 and 1103 in the IPMA. Strains 1121 and 1103 have a proline at position 131 instead of a threonine (T131P) and an arginine instead of a glycine at position 191 (G191R). Proline is known to be a helix breaker and glycine has a great conformational flexibility. Apart from the changes in the primary structure of the protein, T131P and G191R may have important consequences on the secondary and tertiary structure of the protein. Position 131 is located within, and position 191 is located at the outer border of, an antigenic domain (Mahé *et al.*, 2000). Therefore, the substitutions at positions 131 and 191 might be involved in the absence of reaction of mAb 13H4 with strains 1121 and 1103. Previously, it was demonstrated by Meerts *et al.* (2005a) that the production of infectious virus in PK-15 cells is more efficient for Stoon-1010 than for strain 1121.

Fenaux *et al.* (2004) demonstrated that PCV-2 that was passaged 120 times in PK-15 cells (VP120) replicated more efficiently in PK-15 cells than wild-type virus that had been passaged only once (VP1). Differences between VP1 and VP120 were a mutation from proline to alanine at position 110 (P110A) and a mutation from arginine to serine at position 191 (R191S). This may suggest that basic amino acid residues at position 191 influence not only mAb reactivity, but also the production of infectious virus.

Recently, it was demonstrated that PMWS-affected animals are not able to produce neutralizing antibodies, whereas their ability to produce non-neutralizing antibodies remains unaffected (Meerts *et al.*, 2005b, 2006; Fort *et al.*, 2007). In these studies, it was suggested that PMWS-affected animals mount an immune response to non-neutralizing epitopes but not to neutralizing epitopes. In the present study, none of the tested mAbs was able to neutralize all seven PCV-2 strains, suggesting that a universal PCV-2 neutralizing epitope does not exist. Neutralization was observed for Stoon-1010, 48285, 1206 and 1103, but not VC2002, 1147 and 1121. No discriminative amino acid motifs that could explain these results were detected. The mAbs that neutralized Stoon-1010, 48285, 1206 and 1103 did not differentiate these strains from VC2002, 1147 and 1121 in the IPMA, indicating that these two different groups of PCV-2 strains have different neutralizing epitopes, and suggesting that these two different groups of PCV-2 strains use different entry pathways in PK-15 cells. Recently, the glycosaminoglycans heparan sulfate and chondroitin sulfate B have both been described as attachment receptors for PCV-2 (Misinzo *et al.*, 2006). Protein binding to these two attachment receptors is restricted to the basic amino acids lysine and arginine (Esko, 1999), suggesting a crucial role of basic amino acid residues in the entry of PCV-2 into the host cell. The positive amino acid charges of lysine and arginine interact three-dimensionally with negatively charged glycosaminoglycan sulfates and carboxylates (Esko, 1999), which indicates that three-dimensional conformation plays a crucial role in interactions between the PCV-2 capsid protein and its receptors.

Until now, it was assumed that no distinct antigenic variation existed among PCV-2 isolates. In this study, we have clearly demonstrated the existence of major antigenic differences among the capsid proteins of PCV-2 strains with a different genotype and isolated from different clinical presentations.

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REFERENCES

Allan, G. M. & Ellis, J. A. (2000). Porcine circoviruses: a review. *J Vet Diagn Invest* **12**, 3–14.

Allan, G. M., McNeilly, F., Cassidy, J. P., Reilly, G. A., Adair, B., Ellis, W. A. & McNulty, M. S. (1995). Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet Microbiol* **44**, 49–64.

Allan, G. M., McNeilly, F., Kennedy, S., Daft, B., Clark, E. G., Ellis, J. A., Haines, D. M., Meehan, B. M. & Adair, B. M. (1998). Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J Vet Diagn Invest* **10**, 3–10.

Allan, G. M., McNeilly, F., Meehan, B. M., Kennedy, S., Mackie, D. P., Ellis, J. A., Clark, E. G., Espuna, E., Saubi, N. & other authors (1999). Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. *Vet Microbiol* **66**, 115–123.

Allan, G. M., McNeilly, F., Kennedy, S., Meehan, B., Moffett, D., Malone, F., Ellis, J. & Krakowka, S. (2000). PCV-2-associated PDNS in Northern Ireland in 1990. Porcine dermatitis and nephropathy syndrome. *Vet Rec* **146**, 711–712.

Cheung, A. K. (2003). Transcriptional analysis of porcine circovirus type 2. *Virology* **305**, 168–180.

Cheung, A. K., Lager, K. M., Kohutyuk, O. I., Vincent, A. L., Henry, S. C., Baker, R. B., Rowland, R. R. & Dunham, A. G. (2007). Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* **152**, 1035–1044.

Cságola, A., Kecskeméti, S., Kardos, G., Kiss, I. & Tuboly, T. (2006). Genetic characterization of type 2 porcine circoviruses detected in Hungarian wild boars. *Arch Virol* **151**, 495–507.

Dán, A., Molnár, T., Biksi, I., Glávits, R., Shaheim, M. & Harrach, B. (2003). Characterisation of Hungarian porcine circovirus 2 genomes associated with PMWS and PDNS cases. *Acta Vet Hung* **51**, 551–562.

de Boisséson, C., Beven, V., Bigarre, L., Thiery, R., Rose, N., Eveno, E., Madec, F. & Jestin, A. (2004). Molecular characterization of *Porcine circovirus* type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs. *J Gen Virol* **85**, 293–304.

Delputte, P. L., Vanderheijden, N., Nauwynck, H. J. & Pensaert, M. B. (2002). Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *J Virol* **76**, 4312–4320.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Wilson, P., Strokappe, J., Martin, K., McNeilly, F. & other authors (1998). Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can Vet J* **39**, 44–51.

Esko, J. D. (1999). Glycosaminoglycan-binding proteins. In *Essentials of Glycobiology*, pp. 441–453. Edited by A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart & J. Marth. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Fenaux, M., Halbur, P. G., Gill, M., Toth, T. E. & Meng, X. J. (2000). Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to

detect and differentiate between infections with PCV-1 and PCV-2. *J Clin Microbiol* **38**, 2494–2503.

Fenaux, M., Opriessnig, T., Halbur, P. G., Elvinger, F. & Meng, X. J. (2004). Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. *J Virol* **78**, 13440–13446.

Fort, M., Olvera, A., Sibila, M., Segales, J. & Mateu, E. (2007). Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. *Vet Microbiol* (in press). doi:10.1016/j.vetmic.2007.06.004

Galfre, G. & Milstein, C. (1981). Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol* **73**, 3–46.

Grierson, S. S., King, D. P., Wellenberg, G. J. & Banks, M. (2004). Genome sequence analysis of 10 Dutch porcine circovirus type 2 (PCV-2) isolates from a PMWS case–control study. *Res Vet Sci* **77**, 265–268.

Hamel, A. L., Lin, L. L. & Nayar, G. P. (1998). Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol* **72**, 5262–5267.

Hamel, A. L., Lin, L. L., Sachvie, C., Grudeski, E. & Nayar, G. P. (2000). PCR detection and characterization of type-2 porcine circovirus. *Can J Vet Res* **64**, 44–52.

Harding, J. C. S. (1996). Postweaning multisystemic wasting syndrome: preliminary epidemiology and clinical presentation. In *Proceedings of the 27th Annual Meeting of the Western Canadian Association of Swine Practitioners*, p. 21, October 1996. Saskatoon.

Kim, J. H. & Lyoo, Y. S. (2002). Genetic characterization of porcine circovirus-2 field isolates from PMWS pigs. *J Vet Sci* **3**, 31–39.

Knell, S., Willems, H., Hertrampf, B. & Reiner, G. (2005). Comparative genetic characterization of *Porcine circovirus* type 2 samples from German wild boar populations. *Vet Microbiol* **109**, 169–177.

Labarque, G. G., Nauwynck, H. J., Mesu, A. P. & Pensaert, M. B. (2000). Seroprevalence of porcine circovirus types 1 and 2 in the Belgian pig population. *Vet Q* **22**, 234–236.

Ladekjær-Mikkelsen, A. S., Nielsen, J., Storgaard, T., Bøtner, A., Allan, G. & McNeilly, F. (2001). Transplacental infection with PCV-2 associated with reproductive failure in a gilt. *Vet Rec* **148**, 759–760.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.

Larochelle, R., Magar, M. & D'Allaire, S. (2002). Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. *Virus Res* **90**, 101–112.

Lekcharoensuk, P., Morozov, I., Paul, P. S., Thangthumniyom, N., Wajjawalku, W. & Meng, X. J. (2004). Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. *J Virol* **78**, 8135–8145.

Liu, J., Chen, I. & Kwang, J. (2005). Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J Virol* **79**, 8262–8274.

Liu, J., Chen, I., Du, Q., Chua, H. & Kwang, J. (2006). The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis in vivo. *J Virol* **80**, 5065–5073.

Mahé, D., Blanchard, P., Truong, C., Arnauld, C., Le Cann, P., Cariolet, R., Madec, F., Albina, E. & Jestin, A. (2000). Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. *J Gen Virol* **81**, 1815–1824.

Mankertz, A. & Hillenbrand, B. (2001). Replication of porcine circovirus type 1 requires two proteins encoded by the viral *rep* gene. *Virology* **279**, 429–438.

Mankertz, A., Domingo, M., Folch, J. M., Le Cann, P., Jestin, A., Segales, J., Chmielewicz, B., Plana-Duran, J. & Soike, D. (2000). Characterisation of PCV-2 isolates from Spain, Germany and France. *Virus Res* **66**, 65–77.

Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C. & Finsterbusch, T. (2003). New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. *J Virol* **77**, 9885–9893.

Mateusen, B., Sanchez, R. E., Van Soom, A., Meerts, P., Maes, D. G. D. & Nauwynck, H. J. (2004). Susceptibility of pig embryos to porcine circovirus type 2 infection. *Theriogenology* **61**, 91–101.

Matthew, W. D. & Sandrock, A. W. (1987). Cyclophosphamide treatment used to manipulate the immune response for the production of monoclonal antibodies. *J Immunol Methods* **100**, 73–82.

McNeilly, F., McNair, I., Mackie, D. P., Meehan, B. M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S. & Allan, G. M. (2001). Production, characterization and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* **146**, 909–922.

Meehan, B. M., Creelan, J. L., McNulty, M. S. & Todd, D. (1997). Sequence of porcine circovirus DNA: affinities with plant circoviruses. *J Gen Virol* **78**, 221–227.

Meehan, B. M., McNeilly, F., Todd, D., Kennedy, S., Jewhurst, V. A., Ellis, J. A., Hassard, L. E., Clark, E. G., Haines, D. M. & Allan, G. M. (1998). Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* **79**, 2171–2179.

Meehan, B. M., McNeilly, F., McNair, I., Walker, I., Ellis, J. A., Krakowka, S. & Allan, G. M. (2001). Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. *Arch Virol* **146**, 835–842.

Meerts, P., Nauwynck, H., Sanchez, R., Mateusen, B. & Pensaert, M. (2004). Prevalence of porcine circovirus 2 (PCV2)-related wasting on Belgian farms with or without a history of postweaning multisystemic wasting syndrome. *Vlaams Diergen Tijds* **73**, 31–38.

Meerts, P., Misinzo, G., McNeilly, F. & Nauwynck, H. J. (2005a). Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, foetal cardiomyocytes and macrophages. *Arch Virol* **150**, 427–441.

Meerts, P., Van Gucht, S., Cox, E., Vandebosch, A. & Nauwynck, H. J. (2005b). Correlation between type of adaptive immune response against porcine circovirus type 2 and level of virus replication. *Viral Immunol* **18**, 333–341.

Meerts, P., Misinzo, G., Lefebvre, D., Nielsen, J., Bøtner, A., Kristensen, C. S. & Nauwynck, H. J. (2006). Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. *BMC Vet Res* **2**, 6.

Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H. M. & Nauwynck, H. J. (2005). Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J Gen Virol* **86**, 2057–2068.

Misinzo, G., Delputte, P. L., Meerts, P., Lefebvre, D. J. & Nauwynck, H. J. (2006). Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *J Virol* **80**, 3487–3494.

Morozov, I., Sirinarumitr, T., Sorden, S. D., Halbur, P. G., Morgan, M. K., Yoon, K. J. & Paul, P. S. (1998). Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J Clin Microbiol* **36**, 2535–2541.

Nauwynck, H. J. & Pensaert, M. B. (1995). Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. *Arch Virol* **140**, 1137–1146.

Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D. & Paul, P. S. (2000). Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J Gen Virol* **81**, 2281–2287.

Nayar, G. P., Hamel, A. & Lin, L. (1997). Detection and characterization of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *Can Vet J* **38**, 385–386.

Olvera, A., Cortey, M. & Segales, J. (2007). Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. *Virology* **357**, 175–185.

Opriessnig, T., McKeown, N. E., Zhou, E. M., Meng, X. J. & Halbur, P. G. (2006). Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. *J Gen Virol* **87**, 2923–2932.

Pensaert, M. B., Sanchez, R. E., Ladekjær-Mikkelsen, A. S., Allan, G. M. & Nauwynck, H. J. (2004). Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Vet Microbiol* **98**, 175–183.

Pogranichniy, R. M., Yoon, K. J., Harms, P. A., Sorden, S. D. & Daniels, M. (2002). Case–control study on the association of porcine circovirus type 2 and other swine viral pathogens with postweaning multisystemic wasting syndrome. *J Vet Diagn Invest* **14**, 449–456.

Rosell, C., Segales, J., Ramos-Vara, J. A., Folch, J. M., Rodriguez-Arrioja, G. M., Duran, C. O., Balasch, M., Plana-Duran, J. & Domingo, M. (2000). Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. *Vet Rec* **146**, 40–43.

Sanchez, R. E., Nauwynck, H. J., McNeilly, F., Allan, G. & Pensaert, M. B. (2001). Porcine circovirus 2 infection in swine foetuses inoculated at different ages of gestation. *Vet Microbiol* **83**, 169–176.

Sanchez, R. E., Meerts, P., Nauwynck, H. J. & Pensaert, M. B. (2003). Change of porcine circovirus 2 target cells in pigs during development from foetal to early postnatal life. *Vet Microbiol* **95**, 15–25.

Segales, J., Allan, G. M. & Domingo, M. (2005). Porcine circovirus diseases. *Anim Health Res Rev* **6**, 119–142.

Song, Y., Jin, M., Zhang, S., Xu, X., Xiao, S., Cao, S. & Chen, H. (2007). Generation and immunogenicity of a recombinant pseudorabies virus expressing cap protein of porcine circovirus type 2. *Vet Microbiol* **119**, 97–104.

Tiels, P., Verdonck, F., Coddens, A., Ameloot, P., Goddeeris, B. & Cox, E. (2007). Monoclonal antibodies reveal a weak interaction between the F18 fimbrial adhesin FedF and the major subunit FedA. *Vet Microbiol* **119**, 115–120.

Tischer, I., Rasch, R. & Tochtermann, G. (1974). Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl Bakteriol [Orig A]* **226**, 153–167.

Tischer, I., Gelderblom, H., Vetterman, W. & Koch, M. A. (1982). A very small porcine virus with circular single-stranded DNA. *Nature* **295**, 64–66.

Tischer, I., Mields, W., Wolff, D., Vagt, M. & Griem, W. (1986). Studies on epidemiology and pathogenicity of porcine circovirus. *Arch Virol* **91**, 271–276.

Tripathi, L. P. & Sowdhamini, R. (2006). Cross genome comparisons of serine proteases in *Arabidopsis* and rice. *BMC Genomics* **7**, 200.

Wellenberg, G. J., Stockhofe-Zurwieden, N., de Jong, M. F., Boersma, W. J. & Elbers, A. R. (2004). Excessive porcine circovirus type 2 antibody titres may trigger the development of porcine dermatitis and nephropathy syndrome: a case–control study. *Vet Microbiol* **99**, 203–214.

West, K. H., Bystrom, J. M., Wojnarowicz, C., Shantz, N., Jacobson, M., Allan, G. M., Haines, D. M., Clark, E. G., Krakowka, S. & other authors (1999). Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J Vet Diagn Invest* **11**, 530–532.